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14. ABSTRACT

Fish scales display arrays of thousands of colored living cells known as chromatophores. In this study, the use of color changes in isolated fish scales was evaluated as a rapid warning signal for delayed neurotoxic agents. The focus was on detecting delayed effects of organophosphate nerve agents like sarin, though the less toxic diisopropylfluorophosphate (DFP) was used as a simulant. DFP caused rapid and long-lasting scale color changes. These signals were readily visible and quantifiable, especially for the brightly iridescent scale colors. DFP induced color changes in scales at similar dose-sensitivity and about 300 times more rapidly than in standard animal models. Scales thus showed promise as toxicity monitors.

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FINAL REPORT

GRANT #: N00014-96-1-566

PROJECT TITLE: Toward optically monitored cytosensors

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INSTITUTION: Oregon State University

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OBJECTIVE: To develop an optical means of detecting bioactive and neuroactive chemical agents. To measure the effects of cytotoxic agents and neurooxic agents on visible color changes in living cellular arrays.

APPROACH: Modern protective gear and prophylactic drugs improve the odds of surviving the acutely lethal effects of nerve agents, but an exposed person is nonetheless at great risk of developing weeks later a debilitating neurological syndrome known as organophosphate induced delayed neurotoxicity . Whether an environment or an analyte might cause delayed neurotoxicity cannot be rapidly predicted at present since even the most sensitive whole animal exposure models for delayed neurotoxicity take a week or more to develop characteristic symptoms of neurological crippling. However, if fundamental early stages of neurological impairment could be observed at the cellular level it might be possible to construct a sensor device that is a real-time warning system for delayed neurotoxicity. Isolated neuronal cells might be adaptable to this purpose, but other cell types with a basic biochemical similarity to nerve cells might have advantages for use in monitoring systems. The colored cells in fish scales have characteristics that could be ideally suited for this purpose. We therefore asked whether cellular signs predictive of delayed neurotoxicity rapidly appear in the colored living cells of fish scales.

Fish scales are complex organs .They have a layered organization that, like the skull, is descended from the external armor of prehistoric fish. Scales are composed in their deepest layers of a rigid bony matrix. This rigid matrix is wrapped at its protruding end by several skin layers, one of which is a thin sheet of vividly colored cells known as chromatophores. For this ONR sponsored study we mainly used scales taken from West African jewel cichlids (Hemichromis bimaculatus). Under low magnification the chromatophores were seen as blending together into dense two-dimensional arrays of color in much the same way that phosphor spots blend on a color computer monitor. One major group of chromatophores includes three types of pigmented chromatophores: black melanophores, red erythrophores, and yellow xanthophores. The other major group is made up of the iridescently colored iridophores, a type of cell whose color is due not to pigment but to multilayer light reflection. Each scale displayed hundreds of each type of chromatophore. There was an even distribution of melanophores and xanthophores over most of the skin attached to the scale. Erythrophores, whose diameters were large enough to make the individual cells prominent at this magnification, were also distributed fairly evenly. In contrast, the iridophores clustered into one or two brilliant patches about 1 mm in width. Melanophores were mingled in the iridophore patch, but no xanthophores or erythrophores were present there. Reflections from the iridophore patch could be observed in isolation from the other scale colors by placing the scale on a black backing and illuminating it from above.

The main purpose of these studies was to evaluate the feasibility of monitoring environments with scale chromatophores. Many fish naturally change their colors for purposes of camouflage, mating displays, and communication. Color changes in the animal are regulated by both the nervous and endocrine systems via hormones and neurotransmitters that trigger intracellular movements of colored organelles. An example of whole animal color regulation was echoed in vitro when scales were exposed to norepinephrine, an endogenous hormone and neurotransmitter. Within seconds, pigment began to

aggregate toward the centers of melanophores and erythrophores, and to disperse outwardly in xanthophores. As a result of these pigment movements, the erythrophores and melanophores appeared as dark but small points, giving the skin an overall pale complexion. Above this pale background there was a striking norepinephrine-induced change in hue from blue toward yellowish-green in the iridophore patches. This change toward longer reflected wavelengths implied that intracellular movements had caused an increase in the distance between the crystalline platelets of iridosomes.

The organelle movements responsible for color changes in chromatophores are accomplished by fundamentally the same kinds of intracellular machinery that power axonal transport in nerve cells. Axonal transport is the means by which intracellular materials move over long distances along cytoskeletal tracks in nerve cells at speeds ranging from less than a millimeter per day to nearly a meter per day. A well-discussed hypothesis proposes that delayed neurotoxicity results when organophosphates inhibit as yet unknown enzymes to cause interference in the normal functions of the cytoskeleton involved in axonal transport. We therefore tested whether DFP, an organophosphate that causes delayed neurotoxicity, would cause color changes in fish scale chromatophores.

ACCOMPLISHMENTS: The action of DFP on the iridescent color of jewel cichlid scales was extraordinary. The iridophore patch changed from its basal blue color to an extremely vivid yellowish-green color that is similar though brassier than the color induced by norepinephrine. The brassy iridescence induced by DFP concentrations greater than about 200 μM was easily distinguished from the basal blue iridescence by the naked eye from a distance of several feet. With the aid of a dissecting microscope the threshold DFP dose that could be detected by iridescence change was 10 μM. A threshold dose-sensitivity of 10 μM is about the same as the most sensitive whole animal assay for DFP-induced delayed neurotoxicity. The sensitivity of the fish scale to DFP can be expected to increase further by increasing the permeability of the chromatophores to DFP.

Thus, DFP induced iridescent color changes quickly enough and at low enough concentrations to make chromatophores promising tools for environmental monitoring of delayed toxic agents. Indeed, iridescent color change in the iridophore patch was at least 300 times more rapid than the delayed neurotoxic response in standard whole animal models (30 minutes compared to 7 to 14 days).

In addition to causing rapid iridescence changes, DFP also induced pigment movements in melanophores, erythrophores and xanthophores. These pigment movements were in the same general directions as those triggered by norepinephrine. However, DFP caused pigment transport at uneven speeds and in irregular clumps unlike the smooth and even pigment transport induced by norepinephrine (data not shown). That DFP and norepinephrine caused similar (though not identical) changes in iridescence and pigment transport suggested that DFP toxicity mimicked some but perhaps not all aspects of intracellular signal transduction triggered by norepinephrine.

Scales of a distantly related fish from the same taxonomic family as the jewel cichlid, the East African Nile tilapia (Oreochromis niloticus), were also tested for their response to DFP. Though these scales were less iridescent than those of the jewel cichlid, a comparable set of color changes were induced by DFP. Scales of zebrafish (Danio rerio), a popular laboratory model organism from a different family than the jewel cichlid, also changed colors in essentially the manner described above when they were challenged by DFP. The utility of fish scale monitors thus extends to many animal sources. All of these scales had the advantageous qualities of having undiminished sensitivity to DFP over a prolonged storage period (at least two weeks), as well as optimal responsiveness at temperatures ranging ten degrees above and below room temperature (data not shown).

Scales were tested against two other neurotoxic agents. Color changes similar to those just described were induced in jewel cichlid scales by the organophosphate nerve agent paraoxon. Color changes were therefore generalized to at least this additional organophosphate. The effectiveness of paraoxon in inducing color change was low compared to DFP, mirroring what is reported for animal models. In another study, scales of the Nile tilapia were exposed to phenylmethylsulfonylfluoride (PMSF). PMSF is neither an organophosphate, nor a delayed neurotoxicant, but studies in whole animals have shown that PMSF most probably causes early, reversible inhibition of the initiating enzyme(s) in delayed neurotoxicity. It was therefore interesting that PMSF induced color changes that were indistinguishable from those induced by DFP. This suggested that the rapidity of fish scale monitoring

enables early toxic mechanisms to be detected, including those that wane during the long incubation period in whole animal testing.

Tests revealed that DFP-induced color changes in fish scales were not mediated by inhibition of acetylcholinesterase, the enzyme target for the acute effect of nerve agents. Instead, color changes were induced by the action of DFP on yet to be identified targets. Such a noncholinesterase toxic mechanism is also the hallmark of organophosphate-induced delayed neurotoxicity in whole animal models.

The initial characterization of the system described above showed that delayed neurotoxicants can induce color changes in fish scales. We next addressed the question of how DFP caused color changes. Most important was the issue of delayed toxicity: Do delayed neurotoxicants have as long-lasting consequences for chromatophores as they do for nerve cells?

A key finding, therefore, was that the color changes induced by DFP persisted up to days after DFP was removed. This sort of effect is precisely as expected if the intracellular transport machinery in chromatophores becomes lastingly impaired as is hypothesized for organophosphate-intoxicated nerve cells. Such long-lasting color changes in chromatophores have not been seen before to our knowledge. Instead, it is typical for chromatophores to revert to their original color when a stimulus such as norepinephrine is withdrawn. We thus focused on understanding long-lasting color changes by employing a tool, forskolin, that quickly reverses norepinephrine's effects in chromatophores. Forskolin is a plant derivative that activates adenylyl cyclase, the enzyme in cells that manufactures second messenger cAMP. Interestingly, forskolin dramatically reversed cases of DFP-induced iridescence which had lasted for up to 4 days. In contrast, forskolin caused only minor reversals of DFP-induced changes in the pigment-containing chromatophores (melanophores, erythrophores, xanthophores). Thus two types of long-lasting DFP-induced color changes were distinguished by forskolin: one (iridophores) that is quickly reversed by forskolin, and the other (pigmented chromatophores) that is not.

Observations of individual melanophores by time-lapsed video microscopy showed numerous examples of DFP-treated cells in which pigmented organelles migrated fairly normally for short distances, but then bunched up into clumps as though they had reached a bottleneck in the tracks. This suggested that irreversibility of DFP-induced color changes in melanophores by forskolin reflects disruptions in the normal organization of the cytoskeletal tracks along which pigment is translocated. Erythrophores and xanthophores appeared to be impaired by DFP in the same way as melanophores. These abnormal subcellular mechanics in the pigmented chromatophores led to the prediction that DFP would impair the movement of pigment out of any region of a cell to which it was localized at the time the cell was intoxicated by DFP.

To test this prediction of impaired transport, scales were exposed first to norepinephrine, which induced pigment to move into the distinctive distributions causing pale coloration. DFP was then added. Upon removal of the norepinephrine/DFP mixture, none of the chromatophore colors reversed spontaneously. Instead, pigment stayed in position as predicted, and the scale remained pale. Also, the iridophore patch remained brassy. Finally, when the scale was exposed to forskolin, only the color of the iridophore patch was reversed to its basal color (blue). The pigment in the melanophores, erythrophores and xanthophores stayed in place, again fitting the prediction. Closer examination showed that a small degree of grossly impaired transport did take place when forskolin was added, but the distances that the pigment was transported were too small to alter the pale color of the scale. Another experiment tested the above prediction using a converse order of additions. Scales were exposed first to forskolin, followed by addition of DFP. No color changes at all were induced by DFP, which gave the interesting result that forskolin prevented the iridophore patch from being turned brassy as it typically would have with DFP exposure alone. Then, the forskolin/DFP mixture was removed and the scale was challenged with norepinephrine. The scale did not turn pale. Instead, pigment transport was clearly extremely impaired. Indeed, even though norepinephrine caused the iridophores to change hue, the patch was now atypically dark green because of the dark background from the impaired pigmented chromatophores.

CONCLUSIONS: The above findings define a difference between iridophores and the pigmented chromatophores (melanophores, erythrophores, and xanthophores): forskolin protects and rescues iridophores, not the pigmented chromatophores. There was no indication that pigmented chromatophores were killed by DFP. Instead, we suggest that the following sequence of events best explains our observations concerning pigmented chromatophores. First, DFP induces a varying degree of pigment

transport, perhaps by mimicking certain normal signaling mechanisms. Coexposure to norepinephrine or forskolin at this early stage can overpower these initial effects of DFP. But then, most significantly, a long-lasting DFP-induced impairment of transport takes place consistent with disruption of the cytoskeleton. This conclusion that there is impaired pigment transport coincides well with ideas proposed by several research groups implicating an impaired cytoskeleton in delayed neurotoxicity. Various specific mechanisms for cytoskeletal disruption have been put forward by these groups, each of which could be consistent with our findings in pigment-transporting chromatophores.

Iridophores present an added challenge in proposing a mechanism for how DFP causes long-lasting changes since the mechanism needs to account for how the impairment is quickly overcome by treatment with forskolin. The issue is highly interesting because this is among the few, if any, discovered examples of overcoming a long-lasting toxic effect of an organophosphate. The known pharmacological activity of forskolin is to activate adenylyl cyclase and thereby raise intracellular cAMP concentration. This in turn is predicted to cause the activation of cAMP-dependent protein kinase within the iridophore. Therefore a question in need of further research is how presumed cAMP-dependent protein phosphorylation can reverse long-lasting changes in cytoskeleton-dependent transport in iridophores but not in the pigmented chromatophores.

SIGNIFICANCE We have thus found that fish scales are a promising system for detecting organophosphate nerve agents and studying their mechanisms of toxicity. There is evidence that biological toxins can also be detected by chromatophores. For example, chromatophores were found to be sensitive to bacterial cholera toxin, and chromatophores also exhibited increased sensitivity to DFP in the presence of staphylococcal alpha-toxin. Chromatophores may therefore have utility for both chemical and biological toxicity monitoring.

PATENT INFORMATION: A patent application related to using chromatophores to detect bioactive substances has been filed.

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